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FORMATION OF HYDROCARBONS BY BACTERIA AND ALGAE

THOMAS G. TORNABENE

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Solar Energy Research Institute
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1617 Cole Boulevard
Golden, Colorado 80401

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NOTICE

FORMATION OF HYDROCARBONS BY BACTERIA AND ALGAE

Thomas G. Tornabene

Solar Energy Research Institute

Golden, Colorado 80401

The chemical investigation of biologically synthesized hydrocarbons did not begin early in the history of the systematic study of fats. All the neutral or highly non-polar lipids were included in a category of compounds designated as waxes. The waxes were monoesters of fatty acids and long chain alcohols, hydrocarbons, long chain alcohols, and high molecular weight compounds. Systematic investigations into the derivation and chemical nature of the constituents of waxes was started in 1942 by the American Petroleum Institute Project 43 which was designed to determine a) the part played by microorganisms in the formation of petroleum, b) the type hydrocarbons synthesized as animal and plant products to the extent and variety necessary to be able to form crude oil and c) whether radioactive and thermal sources of energy can transform organic matter into petroleum. The rationale for this project was apparently based on a number of factors. In 1899 it was proposed that complex organisms, such as trees, fish and animal fats could be a direct source of the hydrocarbons in petroleum (1). In 1906, the isoprenoid hydrocarbon squalene was isolated as the major constituent of shark liver oil (2-4). Diatom nobs in tertiary opal shales were reported in 1926 (5). These nobs were apparently secreted primarily by diatoms including the hydrocarbons and other organic matter found in them. Based on the field and microscopic studies of sediments and diatom blooms, Becking et al. (6) concluded in 1927 that diatoms directly produce hydrocarbon oils. Trask suggested in 1932 (7) on the basis of a laboratory experiment that bacteria are capable of creating reducing conditions for conversion of organic material into oil. Since these reports, simple organisms such as algae, foraminifera and bacteria were proposed as the sources of the hydrocarbons on the consideration of the age of petroleum (8) and the ability of the microorganism to survive under anaerobic and other adverse conditions (9).

Project 43A under the direction of Zobell investigated the action of bacteria on organic substances and the possible hydrocarbons produced by bacteria as component parts of their cell substance (10-12). In a series of papers it was reported that a) practically all hydrocarbons can be attacked under suitable conditions by some bacterial form (10), b) bacteria can convert caproic acid to hydrocarbons of the C-20 to C-25 range (11), and c) marine bacteria such as *Serratia marinorubra* contain appreciable amounts of liquid and solid hydrocarbon substances (12). It was also speculated that bacteria might contribute to the liberation and migration of oil by destroying organic and inorganic structures in which the oil may be entrapped and by producing emulsion agents, as fatty acids, that enabled oil to migrate. The finding that caproic acid could be converted to hydrocarbons by bacteria was considered a major breakthrough, offering a possible explanation to earlier work in 1931 which had shown fatty acids to be transformed into methane and carbon dioxide (13). In a progress report for A.P.I., Knebel (14) reported that a freshwater sediment contained hydrocarbons, confirming that not all hydrocarbons in recent sediments are destroyed by bacterial action. The most significant part of the report was that hydrocarbons extracted from bacteria and algae exhibited optical properties comparable to the optical activity of the active fractions of petroleum. Nevertheless, no information was obtained concerning the nature and composition of microbial intracellular hydrocarbons. The A.P.I. project was terminated in 1952. Some significant results emerged from this project but no clear, definitive data were generated to support the concept of biotic origin of petroleum hydrocarbons. Scattered reports on microbial production of hydrocarbons continued to appear until 1967 but with emphasis on the hydrocarbons as possible biological markers for studying geologic time and geologic conditions (15).

A resurgence in the biotic theory of origin of petroleum resulted in the mid-1960's from the finding of large amounts of alveolar "yellow bodies" in carboniferous limestone series of the Scottish Lothian (Torbanite). These yellow bodies were identified as the remains of an alga that appeared identical to those from the contemporary alga *Botryococcus braunii*. It was subsequently shown that 80% of the organic material of the brown resting stage of the alga was acyclic hydrocarbon (16,17). "Each cell is embedded in a cup of oil and when a cell divides into two daughter cells the latter secrete oil, while remaining inside the cup of the mother cell. Thus the matrix of the colony is built up of the cups of the daughter cells" (16,18). It was believed that the Torbanite orginated from *B. braunii*.

It is known today that isoprenoid and non-isoprenoid acyclic hydrocarbons are components of most microorganisms. The concentrations of the hydrocarbons, however, vary from only trace constituents to major components of the cellular organic materials.

The hydrocarbon synthesizing capability of microorganisms that produce them as a major constituent are restricted to only few algal, bacterial and fungal species. Individual species that produce hydrocarbons as major components have been isolated from mesophilic, thermophilic, psychrophilic, acidophilic, alkalinophilic and halophilic environments under aerobic or anaerobic, autotrophic or hetertrophic conditions. The environmental distribution of hydrocarbon producers follows no discernible pattern that can be used as a guide for finding prolific hydrocarbon producers.

Hydrocarbons other than squalene generally occur as only trace constituents of marine animals, but may be major components of algae. In a variety of marine and freshwater algae, including a red, greens, browns, diatoms and phytoplankton, the hydrocarbon heneicosahexaene (C-21:6) exists in amounts inversely correlated with the abundance of the long-chain highly unsaturated fatty acid (C-22:6) (19-23). The all cis-3,6,9,12,15,18-heneicosahexaene was first isolated from the diatom *Skeletonema costatum* (24). Since then, the isomer all cis-1, 6,9,12,15,18-heneicosahexaene was reported in a variety of other algae (19-23), diatoms (19-23), and phytoplankton (19-23). It now appears that the Δ^1 isomer is produced by only the brown algae, that the Δ^3 isomer occurs in green algae and diatoms and with one exception, the red algae do not produce significant amounts of these polyunsaturated hydrocarbons (25). The exact structure of the positional isomer of the polyunsaturated hydrocarbon in phytoplankton is unresolved. This polyunsaturated hydrocarbon is produced in quantities exceeding 1% of the total dry weight of some species of brown and green algae. In contrast, nonphotosynthetic diatoms, dinoflagellates, cyanobacteria and photosynthetic bacteria contain traces of aliphatic hydrocarbons, but no C-21:6.

Blumer et al. (26) surveyed microalgae for hydrocarbon content. Their analyses of 23 species of algae belonging to 9 algal classes yielded results similar to those of Lee and Loeblich (19) and Youngblood et al. (27). Lee and Loeblich reported the distribution and quantitation of 21:6 hydrocarbons and 22:6 fatty acid within the major groups of algae in both marine and freshwater environments while Youngblood et al. (27) identified both the saturated and olefinic hydrocarbons of 4 green, 14 brown and 6 red benthic marine algae from the Cape Cod area of Massachusetts, USA (Table I). Their data indicated that n-pentadecane (C-15) predominates in brown algae, n-heptadecane (C-17) in red algae, olefins predominate in green and brown algae, and that polyunsaturated C-19 and C-21 hydrocarbons occur in brown and green algae and in only a few of the red algal species (20,25,27). A C-17 alkyl-cyclopropane was tentatively identified in two species of green algae. Among the unsaturated hydrocarbons, mono- and di-olefinic C-15 and C-17 hydrocarbons were common. Similar data were reported by Shaw and Wiggs (28) for Alaskan marine intertidal algae.

TABLE I

Principal Normal Hydrocarbons In Marine Algae

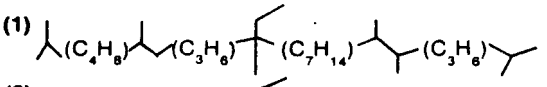
SPECIES	15:0*	15:1	17:0	17:1	17:2	17:3	19:5	19:6	21:5	21:6	23:0	24:0	25:0	26:0
Green Algae														
Enteromorpha compressa				96										
Ulva Lactuca				88					2.2	4.0				
Spongomorpha arcta							9.4	6.7	22.0	60.0				
Codium fragile			89.0	5.9										
Brown Algae														
Ectocarpus fasciculatus	7.0									91.0				
Pilayella littorales										98.0				
Leathesia difformis	59.0		3.6								5.7	13.0	6.0	5.4
Punctaria latifolia	85.0									5.9				
Scytosiphon lomentaria	38.0		11.0	15.0		4.2	19.0							
Chorda filum	38.0		2.3						37.0	10.0				
Chorda tomentosa	31.0						2.5	2.7	17.0	47.0				
Laminaria agardhii	73.0								2.8	3.8	2.4		4.2	5.0
Laminaria digitata	64.0						17.0	9.7						
Ascophyllum nodosum	56.0				23.0	4.6		5.8						
Fucus distichus	98.0													
Red Algae														
Porphyra leucosticta		15.0	17.0				62.0							
Dumontia incrassata			98.0											
Chondrus crispus			99.0											
Rhodymenia palmata			99.0											
Ceramium rubrum			96.0											
Polysiphonia urceolata			96.0											

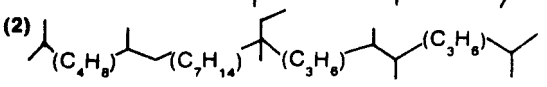
Data taken from Ref. 27; *First number indicates chain length while the second number indicates number of double bonds.

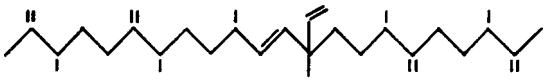
The extremely halophilic green algae are considered important organisms because of their capacity to synthesize glycerol and provitamin A (29-31). The lipids of *Dunaliella salina*, excluding glycerol, comprised some 50% of the cellular organic material; more than 30% of the total lipids consisted of acyclic and cyclic hydrocarbons (32). Carotenes accounted for 21% of the cell mass. Another 3.5% was saturated and unsaturated C-17 straight chain hydrocarbons and internally branched 6-methyl hexadecane and 4-methyl octadecane (32). Methyl branched alkanes, other than the more common iso- and anteiso-structures, are particularly significant because of their restricted occurrence in microorganisms. The internally methyl branched linear alkanes like those identified in *Dunaliella* had previously been reported a unique feature of only the cyanobacteria (33-36).

The chlorophytes, such as *Coelastrum*, *Chlorella*, *Scenedesmus* and *Tetraedron* that are contemporary algae found in sediments contain saturated and unsaturated C-17 components typical of green algae (33). Certain alga, however, contain in addition to C-17, the unsaturated C-27 (*Scenedesmus*) or saturated C-23, C-25 and C-27 (*Tetraedron*) chains (33). These findings are contrary to Han and Calvin's prediction (37) that longer chain hydrocarbons, especially ones that are also major constituents, are absent in algae. The green alga *Botryococcus braunii*, implicated in the formation of tertiary sediments (16), produces unusual hydrocarbons when in particular physiological growth states. *B. braunii* is a freshwater green colonial algae of widespread occurrence which has at least two, and possibly three, physiologically distinct forms. The large green resting cells synthesize negligible amounts of hydrocarbons while the green fast growing cells (exponential growth) produce the unbranched, diunsaturated hydrocarbons heptacosa-1,18-diene, nonacosa-1,20-diene and hentriaconta-1,22-diene as the major components and unsaturated heptadecane, trieicosene and pentaeicosene as the minor constituents. These constituents in total account for approximately 17% of the cellular composition (27,38). Cells that are in a brown resting stage, which often arise as massive rust-colored algal blooms on the surface of lakes, contain two unsaturated isomeric hydrocarbons of the formula $C_{34}H_{58}$. The two components termed botryococcene and isobotryococcene (16) comprise between 70-90% of the cellular composition. Hydrogenation of both hydrocarbons results in the same hydrocarbon botryococcane structure 1 or 2 (16).

Structure 3 was proposed by Cox et al. (39) for botryococcene. Although this alga can produce copious amounts of hydrocarbon oils on a mass basis, an improved culturing system and prescribed growth parameters will have to be developed before any realistic consideration can be given to the employment of this alga as a direct source of hydrocarbon. The enormous difference in hydrocarbon content among the different growth stages of *B. braunii* may have been the origin of discrepancies among earlier reports and suggest the

(1) 

(2) 

(3) 

possibility of inaccuracies that may exist in all previous reports on the evaluation of hydrocarbon biosynthesis in microorganisms when culture age and environmental parameters were not considered.

Cyanobacteria are similar to the green algae in that the concentration of cellular hydrocarbons ranges between 0.02 and 0.15% of the dry weight with the predominant hydrocarbons commonly being C-17 species. They are unlike all other algae, however, except for *Dunaliella*, with respect to the occurrence of linear hydrocarbon chains with an internal methyl branch (33-36,40). The hydrocarbon distribution in cyanobacteria is typically in the carbon range of C-15 to C-19 with the exception of *Anacystis montana* where the hydrocarbon range is from C-17 to C-29 with the major constituents being unsaturated C-25 and C-27 (33). Two blue green bacteria, *Coccochloris elabiens* and *Agmenellum quadruplicatum*, were reported to have neither C-17 nor branched hydrocarbon components but only mono- and diunsaturate C-19 components comprising the hydrocarbon fraction (40). The internally methyl branched linear hydrocarbons are particularly significant because of their limited occurrence. *Chlorogloea fritschii* contains 4-methyl heptadecane (34) similar to that described in *Dunaliella* (32), and 7- and 8-methylheptadecanes like those identified in *Nostoc spp.*, *Anacystis spp.*, *Phormidium luridum*, *Lyngbya aestuarii*, and *Chroococcus turgidus*, (33,34). In addition, 6- and 7-methyl hexadecane were identified in extracts of *C. turgidus* (33).

The n-C-7 hydrocarbon is common to most of the cyanobacteria studied. Since the cyanobacteria are supposedly significant to geochemical evolution, it would seem that geological samples should have an abundant C-17 hydrocarbon content; however, this is not the case. Perhaps, with time, the lighter molecular weight hydrocarbons were preferentially lost leaving the relatively heavier hydrocarbons in place.

Acyclic isoprenoid hydrocarbons are universal but generally exist as minor or trace constituents in cells. However, it is

important to point out that, except for the isoprenoids pristane (C-19), phytane (C-20), and squalene (C-30), acyclic isoprenoid hydrocarbons have been largely ignored in the systematic analysis of fats and oils apparently because of their low quantities in most organisms. Pristane and phytane have been most often sought because of their geochemical significance; but, their distribution is limited in bacteria (41) and they are not found in algae. On the other hand, squalene ($C_{30}H_{50}$), the precursor to sterols, is a triterpene that can be found as a major constituent in some algae (32,33) and it is also widely distributed among bacteria (41-53) as well as in all higher plants and animals. (See Faulkner and Anderson (47) for a representative review on the occurrence of terpenoid hydrocarbons and hydrocarbon pigments in the marine biota).

Although the quantitative data on squalene are few, earlier reports concerning levels in prokaryotes (0.001 to 0.1 mg/g cells) are incorrect. Recent reports of squalene contents indicate *Halobacterium* (42,54) with 1 mg/g of cells, *Methylococcus capsulatus* (48,49) 5.5 mg/g of cells, *Cellulomonas dehydrogenans* (52) 0.5 mg/g of cells, and in the methanogens, *Sulfolobus* and *Thermoplasma* 10 mg/g of cells (44,45). These quantities exceed the squalene concentrations in eukaryotic microorganisms (for example, *Aspergillus nidulans* which contains 0.3 mg/g of cells) (48,49).

In addition to squalene, the neutral lipids of nine species of methanogenic bacteria (including five methanobacilli, two methanococci, a methanospirillum, one methanosarcina as well as two thermoacidophilic bacteria, *Thermoplasma* and *Sulfolobus*,) contained as major components C-25 and/or C-20 acyclic iosprenoid hydrocarbons with a continous range of hydroisoprenoid homologues (44,45). The range of acyclic isoprenoids detected were from C-14 to C-30. Apart from *Methanosarcina barkeri*, squalene and/or hydrosqualene derivatives were the predominant components in all species studied. The components of *M. barkeri* were a family of C-25 homologues (44,45). The structural differences among many of the isoprenoids found in these bacteia, collectively referred to as archaebacteria, is seen in the carbon skeletons of the individual isoprenoids. The carbon skeleton of the C-30 isoprenoid is that expected from a tail to tail (pyrophosphate end to pyrophosphate end) condensation product of two farnesyl derivatives; however a positional isomer of a C-30 isoprenoid that is consistent with a head to tail condensation route was also identified (44,45). The C-25 isoprenoid fraction comprises constituents that result from tail to tail condensations of farnesyl and geranyl derivatives as well as constituents from the condensation of geranyl-geranyl pyrophosphate and one iso-pentenyl pyrophosphate. With the exception of phytane (C-20), the remaining isoprenoids also appear to be synthesized through condensations that involve more than one biosynthetic pathway (44,45). The distribution of the neutral lipid components and their specific variations in relative

concentrations emphasized the differences between the test organisms while the generic nature of the isoprenoid hydrocarbons demonstrated similarities between this diverse collection of bacteria (44,45). The neutral lipid compositions from these bacteria, many of which exist in environmental conditions like those described for the various evolutionary stages of the archean ecology, resemble the isoprenoid distribution isolated from ancient sediments and petroleum (55-60).

Halobacterium cutirubrum, cultivated under aerobic and microaerophillic conditions, contained cellular ratios of squalene to dihydro- and tetra-hydrosqualene that decreased proportionately with decreased aeration rates and lowered growth rates (54). The ratio of squalene to hydrosqualene conversely increased with increased aeration rates (54). Since electron carriers are lipophilic in nature and the squalenes were localized in the cellular subfraction containing the cytochromes, it was assumed that the electron transport carriers and the squalenes were held in close proximity (54).

Small amounts of nonisoprenoid hydrocarbons can be found in extracts from most bacterial cells. However, with appropriate precautions to eliminate extrinsic sources of hydrocarbons from the cultivation, extraction and analytical procedures, it is generally found that hydrocarbon biosynthesis is restricted to a relatively small number of bacteria. Numerous inconsistancies exist among the reports on the hydrocarbon synthesizing capabilities of bacteria. This is due, in part, to several reasons: a) the reporting of uncharacterized or partially characterized mixtures consisting of primarily hydrocarbons; b) the use of organisms that were not adequately identified; c) the employment of cells from vastly different cultivation systems and at different physiological growth stages; and d) the absence of adequate controls and analytical procedures. The most thoroughly studied of the bacterial hydrocarbons have been those from the family *Micrococcaceae*. Kloos et al. (61) have demonstrated, however, that a large percentage of the members in the *Micrococcaceae* were misidentified. Thus, inconsistencies are obvious in the reported determinations of the hydrocarbon contents of micrococcal strains many of which were assumed to be *Micrococcus luteus* (also known as *M. lysodeikticus* or *Sarcina lutea*) (41,61-70). The identities of the hydrocarbons of the micrococci (Table II) in the range from C-16 to C-30 has now been established as families of monounsaturated isomers containing methyl branches in the iso or anteiso or both configurations, symmetrically and asymmetrically disposed on the ends of the isomers (63,67,69). The double bond position is at or near the center of each hydrocarbon chain and some of the gas chromatographically resolved isomers were yet a mixture of positional isomers (67). The identification of the hydrocarbon composition of more than 50 micrococcal species and strains demonstrated that while the generic nature of the hydrocarbons were the same, the carbon distribution ranges were different (61,68).

For example, the major hydrocarbon constituents are C-24, C-25 for *Micrococcus roseus*, C-25, C-26, C-27 for *Micrococcus varians*, C-27, C-28, C-29 for *M. luteus* and C-30, C-31, and C-32 for *Micrococcus sedentarius* (61). The micrococcal species were subsequently differentiated on the basis of the carbon distribution ranges of the cellular hydrocarbons (61). The hydrocarbons of micrococci amount to 20 to 34% of the total lipids (61,66). Aliphatic hydrocarbons are apparently absent in the other members of the taxonomic family *Micrococcaceae* (61,68), which include the staphylococci, planococci and streptococci.

Uncharacterized or partially characterized non-isoprenoid hydrocarbons have been reported in *Pseudomonas spp.* (41,71); *Escherichia coli* (41,72); *Clostridium spp.* (41); *Desulfovibrio spp.* (41,73,74); *Rhodospirillum*, *Rhodopseudomonas*, *Chlorobium* and *Rhodomicrobium* (41); *Chromatium* (75); *Arthrobacter* and *Corynebacterium spp.* (61,70,76); *Mycobacterium* (70); *Vibrio marinus* (77) *Micrococcus spp.* (41,70); *Bacillus spp.* (70); and *Cellulomonas dehydrogenans* (52). The hydrocarbon chain length is generally from C-16 to C-30 and consists of normal alkanes with no predominance of even- or odd-numbered carbon chains, with the exception of *Pseudomonas maltophillia* (71), *Arthrobacter* strain CCM 1647 (61) and *Corynebacterium spp.* ATCC 21183 (61). These bacteria contain methyl branched unsaturated constituents that are similar in the distribution range and chemical configurations to those found in micrococci. For most of the bacteria listed in this group, however, the identities and quantities of hydrocarbons produced (which ranged from a trace to as much as 3% of cell dry weight of some bacteria (see Ref. 70) will have to be confirmed.

The first definitive investigation into the biosynthesis of hydrocarbons was reported by Sanderman and Schweers (78) who demonstrated acetate-^{14}C incorporation into n-heptane by *Pinus jeffreyi*. The n-heptane was the result of a condensation of four acetate units with an apparent decarboxylation. Since this report there have been numerous attempts to understand the biosynthesis of aliphatic hydrocarbons in microorganisms. There is virtually complete agreement that microbial hydrocarbons are derived from fatty acids. Biogenesis and chain elongation mechanism of normal, branched, saturated and unsaturated fatty acids have received intensive study and have been reviewed at frequent intervals (79, 80). The fatty acids converted to hydrocarbons may be either those comprising the cellular lipid pool or those that exist as a separate selective pool. With regard to the respective microbial systems, the hydrocarbons are derived from fatty acids by decarboxylation, elongation-decarboxylation, or decarboxylation-condensation reactions. The fatty acid decarboxylation mechanism in hydrocarbon biosynthesis exists in specific species of cyanobacteria (36), yeast (81), brown algae (19), and zooplankton (82). The decarboxylation and elongation-decarboxylation pathways are supported by

TABLE II

Relative Percentages of the Compositions of C27, C28 and C29 Hydrocarbons of *M. luteus*.

Configuration	Per cent Hydrocarbons		
	C27	C28	C29
Iso-iso´	12.4	15.1	20.6
Anteiso-iso	32.8	22.5	39.6
Anteiso-anteiso´	44.3	--	37.4
Iso-normal	6.9	38.0	2.4
Anteiso-normal	--	24.3	--
Normal	3.6	--	--

labelling experiments (36,81). However, supporting evidence deriving from specific enzyme studies on decarboxylases, oxidases and carboxyl reductases, necessary to reveal the exact mechanism have not yet been described. Hydrocarbon biosynthesis by a carboxyl-end to a carboxyl-end condensation of two fatty acids with one fatty acid under going decarboxylation is well supported in *M. luteus* (83,87). The hydrocarbon biosynthesis in *M. luteus* has been reviewed recently by Albro (83). The selectivity of the fatty acids condensed into hydrocarbons of micrococci are to a degree disproportional to their concentrations in the glyceride lipids (Table III). This indicates the possibility of the existence of a specific pool of fatty acids (63,67,83) and lipid intermediates (83,87). The specific pattern of normal and iso branched even-numbered carbon fatty acids (equations 1 and 2) and iso and anteiso branched odd-numbered carbon fatty acids (equation 2) in micrococci predicts the chemical nature of the ketones (equation 3) or the hydrocarbons resulting from the combination of condensations of the different fatty acids (equation 4). The inhibition of the pathway illustrated by equation 4 by Pb^{2+} results in the synthesis of long chain ketones (equation 3) with branching configurations and carbon numbers

1) Propionic Acid / Acetic Acids → (Acetate) → n-Fatty Acids

2) Leucine, isoleucine or valine → (NH_2) → (CO_2) → (Acetate) → Branched-fatty acid

3) $R\text{-}CH_2\text{-}{*}C(=O)OH + R'\text{-}{}^{+}CH_2\text{-}{\bullet}C(=O)OH \xrightarrow{\bullet\ CO_2} R\text{-}CH\ {*}C(=O)\text{-}{}^{+}CH_2\text{-}R'$

4) $R\text{-}CH_2\text{-}{*}C(=O)OH + R'\text{-}{}^{+}CH_2\text{-}{}^{\bullet}C(=O)OH \xrightarrow{{}^{\bullet}CO_2} C\text{-}CH={*}C\text{-}{}^{+}CH_2\text{-}R'$

that were identical to the corresponding hydrocarbons (89). Normally, the ketones exist as trace constituents of the cellular material. The purified native, specifically radioactively labeled, ketones of *M. luteus* were introduced into a cell free lysate of *M. luteus*. No ketones were converted into hydrocarbons while a fraction of the ketone pool was converted to free fatty acids, some of which were further degraded (89). All lines of evidence support the idea that the ketones are not precursors of hydrocarbons but constituents from the metabolic regulation of the concentration of the cellular fatty acid pool (equation 5).

5) Acetates $\rightleftarrows$ fatty acids $\rightleftarrows$ lipids, ketones, hydrocarbons

The hydrocarbons are an apparent end-product of the regulation of the cellular fatty acid pool. This is supported by the result obtained from the assay for enzymatic activity for hydrocarbon biosynthesis in cell free lysates of *M. luteus* (Figure 1). The fluctuation in the biosynthesis of hydrocarbons in the course of cell growth was a reproducible feature that was not altered by salt precipitation cuts of the lysates to remove possible endogenous inhibitors or by modifications in lysate preparations (89). These data (Figure 1) indicate that the enzymes of the pathways of hydrocarbon biosynthesis is a specifically regulated one. No evidence has been obtained that demonstrates the oxidation and reutilization of the hydrocarbons by *M. luteus*.

In studies on the modes of entry of a palmitic acid chain into an alkane consisting of more than 16 carbon atoms, a suspected intermediate form of the acceptor moiety was identified as a neutral plasmalogen (87,88,90). Studies in this laboratory with exogenous labeled plasmalogens, however, did not support this concept (89). Nonetheless, the direct participation of a neutral plasmalogen or some similar intermediate as that illustrated in equation 6

6) $R\text{-}{}^{+}CH_2\text{-}{*}C(=O)OH + X \rightarrow R\text{-}{}^{+}CH={*}CH\text{-}X + O^{2-} + OH^{-}$

appears essential to form the type of monoene present in *M. luteus* and described in equation 4.

Although it is established that specific microorganisms synthesize hydrocarbons as natural cellular constituents, most microorganisms do not and should not be expected to produce copious amounts of hydrocarbon under natural conditions. Hydrocarbons

TABLE III

Fatty acids of *M. luteus*

Peak No.	Identification
1	i 12:0
2	12:0
3	i 13:0
4	ai 13:0
5	i 14:0
6	14:0
7	i 15:0
8	ai 15:0
9	i 16:0
10	16:0
11	i 17:0
12	ai 17:0
13	18:0
14	18:1

Symbols: i = iso; ai = anteiso. The first number represents the chain length; the second number represents the number of unsaturations.

reside in the hydrophobic regions of cells, namely cellular membranes. It is expected that the enzymes for hydrocarbon biosynthesis are membrane associated and that the biosynthesis occurs at the lipid-water interface. Since there are no apparent cellular transport mechanisms for the secretion of hydrocarbons from cells, the hydrocarbons remain immobilized in the hydrophobic structures of the cells. The cellular burden of hydrocarbons, therefore, must be limited to a relatively small quantity of the total membrane lipids. In addition to the physical-chemical limitations, there are the metabolic energy requirements for hydrocarbon biosynthesis. The biological energy demand to make a fatty acid is given in the stoichiometry of a representative fatty acid synthesis. The hydrocarbons made from the decarboxylation of a fatty acid or condensation of 2 fatty acids with a decarboxylation utilize a large quantity of biological energy. Since the hydrocarbons can not be metabolized by these cells, most organisms apparently do not store their energy reserves as hydrocarbons.

9) 8 Acetyl-CoA + 7 CO_2 + 14NADPH + 14H^+ + 7ATP → palmitic acid

+ 7CO_2 + 8CoASH + 14 $NADP^+$ + 7ADP + 7Pi + 6H_2O

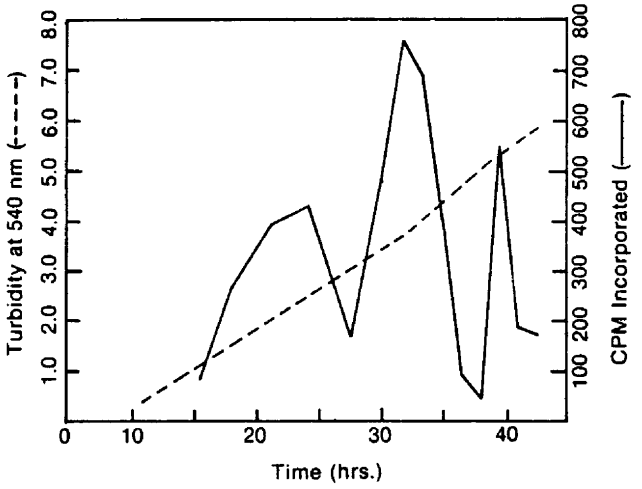


Figure 1. Changes in incorporation of [(^{14}C) U] Palmitic acid into long chain, non-isoprenoid hydrocarbons (————) as a function of growth (--------). At the indicated times 1 x 10^6 cpm of palmitic acid was added to a cell-free extract prepared from an aliquot of cells corresponding to 1 gram dry weight. The cells were grown in 8 liters of Trypticase Soy broth at 25°C with aeration.

Although it is evident that most microorganisms do not synthesize significant quantities of hydrocarbons, there is the obvious exception. *Botryococcus braunii* accumulates hydrocarbons when in the stationary phase of growth in quantities that amount to 80% of its cellular dry weight. These cells embedded in a cup of "oil" release the oil from the cellular matrix when the cells divide. The question that remains is how many other organisms exist that have the equal potential to synthesize hydrocarbon? The current challenge in the field of hydrocarbon bioproduction is to identify the hydrocarbon producing organisms that exist among the immense number of yet "undiscovered" microbes and to determine the genetics and metabolic parameters that control hydrocarbon biosynthesis.

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